

Amendments to the Specification

Please amend the paragraph on page 13, line 1, as follows:

Brief Description of the Figures Drawings

Please amend the paragraph on page 13, lines 3-22, as follows:

Figures 1A-1C

ER-X is neither ER- α nor ER- β . (aA) Western immunoblots of P7 wild-type and ERKO neocortex and adult wild-type mouse ovary, using antibodies to the LBDs of ER- α (Santa Cruz; MC-20; ovary and neocortex) and ER- β (Zymed; ovary). The apparent molecular weight (MW) of mouse ER-X (~62-63 kDa) is clearly different from the MW of the mouse ER- α (67 kDa) and mouse ER- β (60 kDa) ovarian controls. (bB) While P7 wild-type neocortex contained both the 67 kDa ER- α and the ~62-63 kDa ER-X bands, P7 ERKO tissues expressed only the ~62-63 kDa ER-X band. P7 wild-type and ERKO neocortical CLM preparations were greatly enriched with the 62-63 kDa protein. A striking reversal of the ER- α /ER-X ratio was seen in wild-type CLM preparations, in which the 62-63 kDa form was highly enriched, while authentic 67 kDa ER- α was considerably diminished. (eC) Absence of ER- β from the plasma membrane, CLM and non-CLM regions. Note the total absence of ER- β from the ERKO plasma membrane and the CLM and non-CLM fractions. Note also the nuclear concentration of the 60 and 64 kDa isoforms of ER- β . PM, plasma-membrane; non-CLM, non-caveolar-like membrane; CLM, caveolar-like membrane.

Please amend the paragraph on page 13, lines 24-31, as follows:

Figures 2A-2C

Characterization and purity of the CLM preparations. (aA) Western immunoblots of CLMs show enrichment in flotillin, the neuron-specific, integral CLM protein. The purity of CLM preparations was verified (bB) by the presence of caveolar-enriched resident proteins such as PKC- α , and (eC) by the absence of the cytosolic protein paxillin, a cytoskeletal

component associated with non-CLM regions.

Please amend the paragraph on page 14, lines 1-13, as follows:

Figures 3A-3B

ER-X is exquisitely sensitive to picoMolar (pM) concentrations of 17 α -estradiol and 17 β -estradiol. Upper blots: Western immunoblot of ERK1/2 phosphorylation elicited in wild-type neocortical explants by (aA) 17 β -estradiol and (bB) 17 α -estradiol. Lower blots: Re-probing with antibodies to total non-phosphorylated ERK1/2 to verify equal loading of ERK1/2 protein across lanes. (pERK=phosphoERK). Densitometry confirmed equal loading. Note that significantly higher levels of 17 β -estradiol were required for ERK activation, perhaps reflecting the need in wild-type cultures to overcome the inhibitory effect of ER- α on ERK phosphorylation which, unlike 17 α -estradiol, 17 β -estradiol activates as well.

Please amend the paragraph on page 13, line 15 to page 15, line 9, as follows:

Figures 4A-4C

Estrogen-induced activation of ERK1/2 in CLMs and post-nuclear supernatant (PNS). Upper blots: (aA) exposure of highly purified, P7 ERKO neocortical CLMs to 17 α -estradiol (0.1 nM) and 17 β -estradiol (10 nM) for 30 minutes elicited MEK-dependent (U0126) phosphorylation of ERK1 and ERK2 (pERK=phosphoERK). Non-CLM regions were unresponsive. Densitometry confirmed equal loading of protein. (bB) Exposure of P7 wild-type neocortical PNS to 17 α -estradiol (0.1 nM) and 17 β -estradiol (10 nM) for 10 minutes, 37.degree. C. elicited MEK-dependent (U0126) phosphorylation of ERK1 and ERK2. Note that not only did the ER- α -selective ligand PPT reduce ERK phosphorylation levels below baseline (0) very significantly, but the level of ERK1/2 phosphorylation, elicited by 17 β -estradiol, was also significantly lower than following exposure to 17 α -estradiol. This difference may be attributed to the fact that P7 wild-type neocortex is also enriched in ER- α which, since it is activated by 17 β -estradiol (but not 17 α -estradiol), exerts its inhibitory effect on ERK1/2, as was also

seen following exposure to propylpyrazole triol (PPT). Lower blots: Re-probing with antibodies to non-phosphorylated ERK1/2 to verify equal loading of ERK protein across lanes. (pERK=phosphoERK). Densitometry confirmed equal loading. (eC) Densitometric analysis of ERK activation in wild-type PNS shown in (bB). These findings confirm that ER- α is a strong inhibitor of ERK activation, a measure of which is shown by the ability of PPT to effectively prevent ERK activation even in the face of the strong activation of ERK elicited by the PPT vehicle ethanol.

Please amend the paragraph on page 15, line 29 to page 16, line 16, as follows:

Figures 7A-7E

Direct evidence in ERKO that ER-X is a neuronal plasma-membrane-associated receptor with some homology to the ER- α LBD. (aA) Using antibodies highly specific for an alpha-specific region of the LBD of ER- α (C1355), large numbers of immature immunoreactive neocortical ERKO neurons with unstained nuclei are seen. (bB) The immunoreactivity is clearly localized to the cell membrane and cytoplasm, but not in the nucleus. (dD and eE) Antibodies, raised against the full-length ER- α molecule, said to recognize epitopes in the 5', N-terminal region (6F11), but also cross-reacts significantly with ER- β , show widespread nuclear labeling with no cytoplasmic or membrane labeling seen. The nuclear labeling observed most likely reflects intranuclear ER- β which is normally expressed in both wild-type and ERKO neocortical neurons. (eC) CLM association of ER-X in ERKO neocortical neurons was further documented at the ultrastructural level by demonstrating immunoreactive flotillin (gold particles), co-localized with immunoreactivity for the ER- α LBD (horseradish peroxidase) on a mushroom-like neocortical dendritic spine. Scale bars 10 μ m.

Please amend the paragraph on page 16, line 18 to page 17, line 10, as follows:

Figures 8A-8B

Binding of ^3H estradiol to Percoll[®]-purified plasma-membranes from P7 ERKO and wild-type mouse neocortex. (aA) Identical amounts of membrane protein (50 $\mu\text{g}/\text{tube}$) were incubated with varying concentrations of ^3H estradiol (0.3-8 nM) for 18 hours at 4°C. The reaction was terminated by addition of hydroxylapatite (HAP). The membranes and HAP were sedimented by centrifugation in a microfuge, and the pellet washed 4 times to remove free steroid. Radioactivity in the pellets was extracted with ethanol and counted. Non-saturable binding, assessed in the presence of 1 μM unlabelled DES, was subtracted from the total counts and the saturable binding plotted as the ratio of bound/unbound ligand versus the concentration of bound ^3H estradiol. Similar concentrations of high affinity binding (equilibrium dissociation constant, K_d , ~1.6 nM) were observed in wild type and ERKO membranes. (bB) Specificity of the binding site in Percoll[®]-purified membranes from P7 ERKO mouse neocortex. Aliquots of plasma-membrane were incubated with 2 nM ^3H estradiol for 18 hours at 4°C in the presence and absence of different concentrations (50 nM and 1 μM) of 17 α -estradiol, 17 β -estradiol or progesterone. Bound ^3H estradiol was separated by sedimentation with HAP and counted at an efficiency of 50%. Data represent the number of bound counts (after subtraction of HAP-only blank control tubes, containing no membrane protein) expressed as the means +/-S.D. of triplicate determinations. The horizontal dashed line indicates the level of non-specific binding observed in the presence of 1 μM DES.

Please amend the paragraph on page 17, lines 12-18, as follows:

Figures 9A-9B

ER-X is developmentally regulated. ER-X expression is developmentally regulated and is maximally expressed around P7-10 in (aA) the neocortex and (bB) the uterus. During the first postnatal month, wild-type and ERKO neocortical ER-X levels decline dramatically and become barely visible in the adult.

Please amend the paragraph on page 31, lines 10-28, as follows:

Genotyping. Tail snips were obtained from P3-4 pups and used for genotyping, as previously described (Singh, 2000). Briefly, tissues were digested with Proteinase K at 56°C for 90 minutes, followed by a 99°C incubation for 10 minutes. The samples were then vortexed vigorously and insoluble material pelleted in a microfuge. Supernatants were used in a PCR reaction that utilized one primer pair (primer 1: 5'-CGG TCT ACG GCC AGT CGG GCA TC-3' (SEQ ID NO:1); primer 2: 5'-GTA GAA GGC GGG AGG GCC GGT GTC-3' (SEQ ID NO:2)) for the ER-1 gene product (product size=239 base pairs (bp)), and one primer pair (primer 2 from above with NEO Primer: 5'-GCT GAC CGC TTC CTC GTG CTT TAC-3' (SEQ ID NO:3)) for the neomycin insert-containing gene product (product size=790 bp). The PCR program was carried out as follows: 1 cycle at 94°C for 3 minutes, 30 cycles of 94°C for 45 seconds, 62°C for 1 minute, 72°C for 1 minute 40 seconds, followed by a final extension cycle of 72°C for 7 minutes. Products were analyzed by agarose gel electrophoresis. Wild-type animals revealed the smaller 239 bp band, homozygous knockouts (ERKO) showed the larger 790 bp band, and heterozygotes displayed both bands.